Small RNA Functions Are Required for Growth and Development of *Magnaporthe oryzae*

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RNA interference (RNAi) is conserved in eukaryotic organisms, and it has been well studied in many animal and plant species and some fungal species, yet it is not well studied in fungal plant pathogens. In the rice blast fungus *Magnaporthe oryzae*, we examined small RNA (sRNA) and their biogenesis in the context of growth and pathogenicity. Through genetic and genomic analyses, we demonstrate that loss of a single gene encoding Dicer, RNA-dependent RNA polymerase, or Argonaute reduces sRNA levels. These three proteins are required for the biogenesis of sRNA-matching genome-wide regions (coding regions, repeats, and intergenic regions). The loss of one Argonaute reduced both sRNA and fungal virulence on barley leaves. Transcriptome analysis of multiple mutants revealed that sRNA play an important role in transcriptional regulation of repeats and intergenic regions in *M. oryzae*. Together, these data support that *M. oryzae* sRNA regulate developmental processes including, fungal growth and virulence.

RNA interference (RNAi) is a conserved mechanism of eukaryotic gene regulation in which small noncoding RNA (sRNA) mediate posttranscriptional and transcriptional silencing; these sRNA are approximately 20 to 30 nucleotides (Lee et al. 2010). RNAe III-like endonucleases called Dicers successively cleave double-stranded RNA (dsRNA) into sRNA that are loaded into Argonaute (AGO)-containing complexes. The sRNA-loaded complexes, known as RNA-induced silencing complexes (RISC), guide the cleavage of target transcripts or direct the inactivation of homologous genomic sequences (Drinnenberg et al. 2009). In some organisms (namely, plants and fungi), RNAi involves amplification mediated by an RNA-directed RNA polymerase (RdRP); these proteins function to generate secondary small interfering RNA (siRNA) (Baulcombe 2004; Calo et al. 2012; Sijen et al. 2001). Closely related to the role of sRNA in posttranscriptional and transcriptional gene silencing are their functions in cellular processes such as

transposon silencing, viral defense, DNA elimination, and heterochromatin formation (Baulcombe 2004; Bühler et al. 2008; Tomari and Zamore 2005).

Components of RNAi are evolutionarily conserved among many Ascomycete, Basidiomycete, and Zygomycete fungi whose genomes are available, with several exceptions, including *Ustilago maydis* and budding yeasts (Drinnenberg et al. 2009; Nakayashiki 2005). Genomic analysis revealed the presence of Dicer-like (DCL) proteins and, typically, three AGO and three RdRP in ascomycete and basidiomycete fungal genomes. Recent work has focused on molecular analysis of these silencing pathways and their biological roles, revealing differences among fungi. For example, in *Neurospora crassa*, QDE1, DCL-1, and DCL-2 are important for siRNA production in the process of quelling, one of the first-known RNAi-related phenomena (Fulci and Macino 2007; Romano and Macino 1992). In *Cryphonectria parasitica*, a Dicer protects against viral infection (Zhang and Nuss 2008). In the Zygomycete *Mucor circinelloides*, one of the RdRP and Dicer2 are involved in the biogenesis of siRNA (Nicolas et al. 2010). The *Schizosaccharomyces pombe* genome encodes a single gene each for AGO, Dicer, and RdRP (Bühler et al. 2008) and these proteins are involved in heterochromatin assembly (Lee and Collins 2007). RNA silencing in *Aspergillus nidulans* is independent of RdRP (Hammond and Keller 2005).

The ascomycete fungus *Magnaporthe oryzae* causes the most serious disease of cultivated rice, rice blast. The fungus infects additional cereals, including barley and rye, and the distinct Triticum population infects wheat, making it a major threat to global food security (Wilson and Talbot 2009). *M. oryzae* and its rice host have become a model pathosystem in which to study the molecular basis of pathogenesis because of (i) scientific advancements made with both the fungus and the host and (ii) its economic global importance (Dean et al. 2012, Donofrio et al. 2014). The genome of *M. oryzae* contains two *DCL* genes, three *AGO* genes, and three *RdRP* genes (Kadotani et al. 2003; Murphy et al. 2008; Nakayashiki 2005). The *M. oryzae* *DCL2* gene (*MoDcl2*) but not *MoDcl1* is necessary for siRNA production from double-stranded hairpin RNA; this is unlike *N. crassa*, in which the two Dicers act redundantly (Catalanotto et al. 2004; Kadotani et al. 2004). The analysis of sRNA in *M. oryzae* has identified methylguanosine-capped and polyadenylated sRNA (Gowda et al. 2010) as well as sRNA matching repeats, intergenic regions (IGR), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear (snRNA), and protein-coding genes (Nunes et al. 2011; Raman et al. 2013).

sRNA components of several plant-pathogenic fungal species have been characterized (Chen et al. 2014; Mueth et al.

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2015; Yang 2015). In *Fusarium graminearum*, roles have been established for FgAgo1 and FgDicer2 in hairpin-RNA-mediated gene silencing, and FgDicer2 in sRNA transcription and micro RNA (miRNA)-like sRNA (milRNA) generation (Chen et al. 2015). *Botrytis cinerea*, which causes gray mold disease, transfers virulent sRNA effectors into host plant cells, contributing to infection by hijacking the host RNAi machinery and selectively silencing host genes involved in immunity. The *B. cinerea* genome contains two *DCL* genes, and deletion of both results in reduced virulence due to the absence of plant immune-suppressing *B. cinerea* sRNA (Weiberg et al. 2013). We previously characterized sRNA of *M. oryzae* during fungal physiological stress as well as under in planta conditions; we reported that deletion of Dicers (MGG_01541 and MGG_12357) and an RdRP (MGG_13453) affected mRNA (messenger RNA) levels of a subset of fungal genes (Raman et al. 2013). To better understand sRNA biogenesis in *M. oryzae* and to elucidate the role of sRNA in fungal gene regulation and pathogenicity, we have now generated targeted deletion mutants for eight genes involved in sRNA biogenesis and subjected these mutants to both sRNA and mRNA sequencing (RNA-seq). Here, we examined the involvement of these genes in production of sRNA, gene regulation, and pathogenicity. Our results indicate that MGG_12357 (MoDcl2), MGG_13453 (MoRdRP2), and MGG_01294 (MoAgo3) are primarily involved in generation of sRNA. We also report that this same AGO, along with an RdRP, is involved in fungal virulence through an as-yet-undefined mechanism, resulting in reduced sporulation.

RESULTS

RNAi genes of *M. oryzae* are closely related to those of *N. crassa*.

The *M. oryzae* genome encodes two Dicers, three RdRP, and three AGO (Nakayashiki 2005). A search of the most recent annotation, version 8, of the *M. oryzae* genome (released in 2011) at the Broad Institute website revealed similar findings, including two Dicers, three AGO, and three RdRP, in keeping with previous studies (Nakayashiki 2005). One exception to the prior results is the lack of one AGO (MG10003), which no longer exists in version 8; however, a search of the genome for the PIWI, PAZ, and DUF1785 domains, typical of AGO proteins, still yielded three potential AGO. RdRP and AGO are referred to as MoRdRP1 to -3 and MoAgo1 to -3, respectively, by similarly following the naming convention of Kadotani and associates (2008) for Dicers (Supplementary Table S1). Dicer genes include MGG_01541.7 and MGG_12357.7, named *MoDcl1* and *MoDcl2*, respectively, after Kadotani and associates (2008). RdRP include MGG_02748.7 (*MoRdRP1*), MGG_13453.7, and MGG_06205.7, the latter referred to as *MoRdRP2* and *MoRdRP3* in this study. AGO include MGG_14873.7, MGG_13617.7, and MGG_01294.7, named *MoAgo1*, *MoAgo2*, and *MoAgo3* in this study. Phylogenetic analysis of sRNA genes of different fungal species revealed that *M. oryzae* sRNA genes are most closely related to those of *N. crassa* (Fig. 1). Similar to *N. crassa*, the MoDcl1 amino acid sequence lacks the PAZ domain. Unlike *N. crassa*, MoDcl2 has a Dicer dimer domain instead of a dsRNA-specific ribonuclease

Fig. 1. Phylogenetic analysis of A, Dicer; B, RNA-directed RNA polymerase (RdRP); and C, Argonaute (AGO) proteins of fungal species. Sequences were aligned using the ClustalX program with the default values, and the tree was drawn using the MEGA4 program. Species are designated by a two-letter abbreviation preceding the name of each protein: Mo = *Magnaporthe oryzae*, Nc = *Neurospora crassa*, Mc = *Mucor circinelloides*, and Cp = *Cryphonectria parasitica*. Accession numbers are following: MoMDL1 = MGG_01541.7, MoMDL2 = MGG_12357.7, NcSMS-3 = NCU08270, NcDCL-2 = NCU06766, SpDCR1 = SPCC188.13c, McDCL-1 = AM269947, McDCL-2 = FN421463, CpDCL-1 = DQ186989, CpDCL-2 = DQ186990, MoRdRP1 = MGG_02748.7, MoRdRP2 = MGG_13453.7, MoRdRP3 = MGG_06205.7, NcQDE-1 = NCU07534.1, NcSAD-1 = NCU02178.1, NcRRP-3 = NCU08435.1, SpRDP1 = SPAC6F12.09, McRdRP-1 = 111871, McRdRP-2 = 104159, CpRDR1 = 270014, CpRDR2 = 35624, CpRDR3 = 10929, CpRDR4 = 339656, MoAGO1 = MGG_14873.7, MoAGO2 = MGG_13617.7, MoAGO3 = MGG_01294.7, NcQDE-2 = NCU04730, NcSMS-2 = NCU09434, SpAGO1 = SPCC736.11, McAGO-1 = 104161, McAGO-2 = 195366, McAGO-3 = 104163, CpAGL1 = GQ250184, CpAGL2 = GQ250185, CpAGL3 = GQ250186, and CpAGL4 = GQ250187. RdRP and AGO sequences of *M. circinelloides* and sequences of *C. parasitica* RdRP were retrieved from JGI MycoCosm (v 2.0).

domain. The *QDE1* and *QDE2* genes of *N. crassa* are involved in transgene-induced posttranscriptional gene silencing or quelling (Lee et al. 2010), which are closely related to *MoRdRP2* (MGG_13453.7) and *MoAgo3* (MGG_01294.7), respectively. Their involvement in the biogenesis of other types of sRNA is documented (Lee et al. 2010). Based on the phylogenetic analyses, we hypothesized the involvement of *MoDcl1*, *MoDcl2*, *MoRdRP2*, and *MoAgo3* in the endogenous RNAi pathway of *M. oryzae*.

sRNA profiling of *M. oryzae* RNAi mutants and absence of miRNA.

To understand the impact of various RNAi genes on the biogenesis of sRNA, several deletion mutant strains of sRNA biosynthetic genes were constructed using gene replacements and confirmed by Southern analysis (Supplementary Fig. S1). sRNA libraries for these mutants were constructed and sequenced. In our previous study (Raman et al. 2013), sRNA libraries were size selected for 18 to 30 nucleotides (nt). In the current study, low molecular weight RNA were precipitated and used directly for library construction. Based on their similarity to *N. crassa* Dicers, we speculated that there would be redundancy in the roles of *MoDcl1* and *MoDcl2* in sRNA biogenesis. Therefore, we generated double-Dicer knockout mutants. We first generated single-Dicer mutants $\Delta modcl1$ and $\Delta modcl2$ and, subsequently, deleted the remaining Dicer gene, either *MoDcl2* or *MoDcl1*, respectively, resulting in two double-knockout lines, referred to as $\Delta modcl1/2$ and $\Delta modcl2/1$. Two experiments were performed to ensure comprehensive sequencing of all mutants. Due to advances in sequencing and pooling strategies, each individual mutant library from experiment I resulted in 1 to 4 million reads whereas each from experiment II resulted in 14 to 37 million reads. These reads were processed as explained in our previous article (Raman et al. 2013), which revealed the presence of 100,000 to 13,000,000 genome-matched reads; 46,000 to 649,000 distinct genome-matched reads; and approximately 64,000 to approximately 576,000 tRNA-, rRNA-, or small nucleolar RNA (snoRNA)-matched reads (Supplementary Table S2).

Analysis of the distribution of sRNA across various genomic elements in the wild type showed that sRNA-generating loci included matches to protein-coding genes, antisense to protein-coding genes, IGR, repeats, mitochondrial sequences, tRNA and rRNA, and antisense to tRNA and rRNA. As reported earlier, the number of reads matching IGR exceeded the number of reads matching repeats and genes (Fig. 2B) (Gowda et al. 2010; Nunes et al. 2011; Raman et al. 2013).

MoDcl2, *MoRdRP2*, and *MoAGO3* are the main components of sRNA biogenesis in *M. oryzae*.

In order to ascertain the contribution of RNAi genes toward biogenesis of sRNA, a static clustering approach was carried out, as described previously (Raman et al. 2013), to calculate the hits-normalized abundance of all the sRNA reads in each 500-bp cluster of the genome across various mutants, in

comparison with the wild type. If a particular cluster had reads 10-fold or more differentially regulated compared with the wild type, then that cluster was considered to be a differentially regulated cluster (DRC). The distribution of downregulated DRC across the entire genome for each mutant is shown in Table 1. In

Fig. 2. Small RNA (sRNA) profiling of the *Magnaporthe oryzae* wild type and Dicer mutants. **A**, Proportion of total genome-matched sRNA (excluding transfer RNA [tRNA]-, ribosomal RNA [rRNA]-, small nuclear RNA [snRNA]-, and small nucleolar RNA [snoRNA]-matched reads) in each size class for the wild type and mutant libraries. Total genome-matched reads of $\Delta modcl2$, $\Delta modcl2/1$, and $\Delta modcl1/2$ showed different size distribution compared with the wild type. **B**, Distribution of sRNA reads of the wild type and mutants among different genomic elements normalized to tRNA-associated sRNA reads; nt = nucleotides. **C**, 5' Nucleotide preference for sRNA in the *M. oryzae* wild type and mutant libraries. **D**, 5' Nucleotide preference for sRNA matching repeats in the *M. oryzae* wild type and mutant libraries.

total, 113 DRC were found to be Dicer dependent. Most of these loci were distributed among protein-coding gene regions, repeat regions, and IGR. Among the Dicer mutants, the double-Dicer mutants had more DRC than the single mutants. Therefore, we queried whether *MoDcl1* and *MoDcl2* are functionally redundant. To achieve this goal, we calculated the fold changes in read numbers for each of these DRC in the Dicer mutants. The results indicated that most of the Dicer-dependent loci were downregulated between 7- and 10-fold or ≥ 10 -fold in $\Delta modcl2$, whereas they were mostly less than 2-fold downregulated in $\Delta modcl1$. This observation suggests that *MoDcl1* and *MoDcl2* have at least some nonredundant functions and *MoDcl2* is the primary Dicer involved in sRNA biogenesis in *M. oryzae* (Supplementary Fig. S2).

In the case of RdRP, deletion of *MoRdRP3* had no impact and *MoRdRP1* had minimal impact on sRNA generation, whereas *MoRdRP2* had maximum impact on sRNA, demonstrated by a higher number of DRC in the $\Delta morderp2$ mutant. The majority of DRC emanating from this mutant were distributed between LTR (long terminal repeats) and GYPSY (a class of LTR retrotransposons) followed by IGR. Approximately 31 DRC matched genic regions, comprising 16 genes. Similar to the RdRP, deletion of two of the AGO had minimal impact on sRNA generation whereas deletion of *MoAgo3* yielded more DRC distributed between LTR and GYPSY and genic regions, followed in rank order by IGR (Table 1).

Among repeat-associated DRC, the majority were associated with retrotransposons; namely, LTR, GYPSY, LINE (long interspersed nuclear element), and TAD1 (LINE-like elements). Many DRC associated with these retrotransposons were silenced by *MoDcl2*, *MoRdRP2*, and *MoAgo3* deletion. Only five to six DRC associated with DNA transposon (DNA/TcMarPogo) were detected in $\Delta morderp2$ and $\Delta moago3$ mutants, and in other mutants (Table 1). Careful analysis of LTR- and GYPSY-associated DRC downregulated in $\Delta modcl2$, $\Delta morderp2$, and $\Delta moago3$ (Supplementary Fig. S3) indicated the involvement of *MoRdRP2* along with *MoDcl2* or *MoAgo3* in the silencing of the majority of LTR and GYPSY repeats, and the rest of the LTR and GYPSY do not require *MoRdRP2* for silencing. Taken together, our results indicate that *MoDcl2*, *MoRdRP2*, and *MoAgo3* are the primary genetic components involved in sRNA generation in *M. oryzae*.

MoDcl2 is the primary Dicer involved in biogenesis of sRNA in *M. oryzae*.

Size distribution of sRNA sequenced from each RNAi deletion mutants showed that the size classes 19 to 20 and 21 to 23 nt were affected in the $\Delta modcl2$ and double-Dicer mutants (Fig. 2A). This reduction in different size classes of sRNA was

reflected across different genomic elements (Supplementary Fig. S4).

The sRNA profiles of the wild type (strain 70-15, from which all mutants derived) and $\Delta modcl1$ appeared similar. However, the percentage of reads matching IGR and repeats in the $\Delta modcl2$ and the double-Dicer mutants differed from the wild type. A greater number of reads as well as upregulated DRC associated with protein coding genes, tRNA, and rRNA in $\Delta modcl2$ and double-Dicer mutants was likely due to the reduction in reads associated with IGR and repeats (Fig. 2B; Supplementary Table S3). Loss of RDR2 in the model plant *Arabidopsis thaliana* leads to a reduction in repeat-associated reads and an increase in gene-associated reads, as well as a marginal increase in reads associated with IGR (Lu et al. 2006). Because of the abundance of IGR-associated sRNA in our datasets, we checked all IGR or any other regions with characteristics of miRNA-generating loci such as encoded stem-loop structures using the software miREAP. The analysis yielded several predicted stem-loop structures but none had enough read numbers to be considered as candidate miRNA loci, consistent with prior reports from Gowda and associates (2010) and Nunes and associates (2011), indicating an absence of miRNA in *M. oryzae*.

Our previous report (Raman et al. 2013) and Figure 2C show that 5'-U (uracil) sRNA preferentially accumulated in the wild type (e.g., almost half of the sRNA). Deletion of the *MoDcl2* gene disrupted this pattern and increased the accumulation of sRNA with an A in the 5' position. Deletion of both Dicers had a synergistic effect on the increase in 5'-A sRNA. Reduction in 5'-U sRNA by deletion of *MoDcl2* and both Dicers was observed across various genomic elements (Fig. 2D; Supplementary Fig. S5) as well as different size classes of sRNA (although, for unknown reasons, the 20-nt sRNA retained a predominance of 5'-U sRNA; data not shown). This demonstrates the dependency of 5'-U sRNA on *MoDcl2* processing. Size distribution of sRNA across various genomic elements in different mutants showed that the 20-nt peak observed in the $\Delta modcl2$ mutant was not prominent in protein coding sense and antisense, was slightly prominent in IGR, and was more prominent in repeats (Supplementary Fig. S6).

MoRdRP2 is involved in biogenesis of differently sized sRNA.

A reduction in 21- to 23-nt sRNA was observed in the $\Delta morderp2$ mutant (Fig. 3A). This reduction was observed in both protein-coding loci (sense and antisense) and in IGR (Supplementary Fig. S7). Similar to $\Delta modcl2$ and the double-Dicer mutants, deletion of *MoRdRP2* altered the percentage of

Table 1. Distribution of downregulated small RNA clusters in mutants across various genomic loci

Library ^a	Total ^b	With genes ^c	Assoc. ^d	Clusters with tRNA	tRNA (n)	LTR and		LINE and TAD1	DNA/TcMar-Pogo	SR ^e	IGR ^f
						GYPSY					
$\Delta modcl1$	2	2	1	0	0	0	0	0	0	0	0
$\Delta modcl2$	31	13	14	0	0	13	9	0	0	0	4
$\Delta modcl1/2$	89	45	40	0	0	21	11	0	0	0	22
$\Delta modcl2/1$	65	14	13	0	0	24	4	0	0	0	28
$\Delta morderp1$	4	4	3	0	0	0	0	0	0	0	0
$\Delta morderp2$	785	31	16	0	0	459	1	6	0	0	316
$\Delta morderp3$	0	0	0	0	0	0	0	0	0	0	0
$\Delta moago1$	2	2	1	0	0	0	0	0	0	0	0
$\Delta moago2$	1	1	1	0	0	0	0	0	0	0	0
$\Delta moago3$	175	52	29	1	1	89	7	5	2	2	27

^a Libraries compared with the wild type library.

^b Total clusters.

^c Clusters with genes.

^d Number of genes associated.

^e Simple repeats.

^f Intergenic regions.

reads that matched IGR and repeats (Fig. 3B), which in turn increased the percent of reads that matched protein coding genes, tRNA, and rRNA, as well as the number of upregulated DRC (Fig. 3B). Preferential accumulation of 5'-U-containing sRNA was affected by loss of *MoRdRP2*, which is evident from the graph showing reduction in 5'-U sRNA among the total sRNA (Fig. 3C) and sRNA arising from repeat regions (Fig. 3D). These results confirmed the involvement of *MoRdRP2* in sRNA biogenesis predicted from cluster analysis (Table 1).

Loss of *MoAgo3* promotes accumulation of 21- to 23-nt sRNA.

A comparison of the wild type and the AGO mutants revealed a peak at 21 to 23 nt in the $\Delta moago3$ mutant (Fig. 4A). Major changes observed in this mutant for sRNA arising from different genomic elements are (i) increased intergenic and repeat associated sRNA and (ii) a decrease in sRNA originating from both sense and antisense orientations of protein-coding genes (Fig. 4B). An accumulation of 22 mers was observed in the $\Delta moago3$ mutant across various genomic elements, including repeats (Supplementary Fig. S8). There was no difference observed between the wild type and RNAi mutants in preferential accumulation of 5'-U sRNA among total and protein-coding loci (Fig. 4C). For sRNA antisense to protein-coding loci, preferential accumulation of 5'-U sRNA and a reduction in 5'-A sRNA was observed and, for sRNA matching IGR and repeats, a slight reduction in 5'-U sRNA was observed in the $\Delta moago3$ mutant (Fig. 4D). The slight reduction in 5'-U sRNA associated with IGR and repeats indicated a possible reduction in stability of sRNA in the absence of AGO. In summary, our data showed that *MoAgo3* is important but not essential for biogenesis of sRNA. It also has a possible role in the downstream functioning of sRNA, as has been observed in *N. crassa* (Catalanotto et al. 2002) and *S. pombe* (Sigova et al. 2004).

RNAi genes affect the growth, development, and pathogenicity of the fungus.

To understand the role of RNAi genes in growth and development, we tested the growth of the wild type and mutants on complete media plates exposed to different light regimens. Growth of the mutants and the wild type on oatmeal agar (OM) with alternating light and dark conditions for conidial development revealed that fungal growth was significantly affected in the $\Delta morderp1$ and $\Delta moago3$ mutants (Fig. 5A and B). Additionally, $\Delta morderp1$ and $\Delta moago3$ were visibly less pigmented versus the wild type and the other mutants (Fig. 5A). Given that $\Delta morderp1$ and $\Delta moago3$ showed reduced growth and less pigmentation on OM plates, we collected conidia from the wild type and mutants after 10 days on OM plates. The most striking finding was that conidiation was reduced in all mutants, including those with no apparent growth reduction on OM plates (Fig. 5C). The reduction in conidial formation was more pronounced in $\Delta morderp1$ and $\Delta moago3$ followed by $\Delta modc12$, $\Delta modc11/2$, and $\Delta modc12/1$. Mutants $\Delta moago1$, $\Delta morderp3$,

$\Delta modc11$, and $\Delta moago2$ also showed reduced conidiation. These results indicate a role for RNAi in the growth and development of the fungus.

Because both $\Delta morderp1$ and $\Delta moago3$ had significantly reduced conidia production, we were unable to collect enough

Fig. 3. Small RNA (sRNA) profiling of the *Magnaporthe oryzae* wild type and RNA-directed RNA polymerase mutants. **A**, Proportion of total genome-matched sRNA (excluding transfer RNA [tRNA]-, ribosomal RNA [rRNA]-, small nuclear RNA [snRNA]-, and RNA-matched reads) in each size class for the wild type (Wt) and mutant libraries. Total genome-matched reads of $\Delta morderp2$ showed different size distribution compared with the wild type. **B**, Distribution of sRNA reads of the wild type and mutants among different genomic elements normalized to tRNA-associated sRNA reads; nt = nucleotides. **C**, 5' Nucleotide preference for sRNA in the *M. oryzae* wild type and mutant libraries. **D**, 5' Nucleotide preference for sRNA matching repeats in the *M. oryzae* wild type and mutant libraries.

spores to undertake pathogenicity assays using traditional spore suspensions. Therefore, we performed the pathogenicity test via a detached-leaf assay, using mycelial plugs on barley leaves. Barley leaves were wounded via a small, sterile pinprick, allowing mycelia to gain entrance and colonize. The results showed that the

Δmordr1 and *Δmoago3* mutants were unable to infect whereas the other mutants and the wild type were able to colonize and form blast lesions (Fig. 5D). The inability of *Δmordr1* and *Δmoago3* to colonize and cause disease indicate that these genes contribute to virulence.

Pathogenicity genes are differentially regulated in RNAi mutants.

We have observed and demonstrated involvement of RNAi biogenesis genes in sRNA production as well as the growth, development, and pathogenicity of the fungus. We further profiled and compared the mRNA transcriptomes of the wild type and deletion mutants to determine the underlying causes of phenotypes observed, and whether mRNA levels were correlated with sRNA abundance changes in the different mutants. All mutants were subjected to Illumina RNA-seq analysis, which identified 706 differentially expressed genes, comprising of 279 upregulated and 457 downregulated genes (Table 2). It is evident from a heat map of differentially expressed genes that *Δmoago3* and *Δmordr1*, the two mutants that also demonstrate pathogenicity defects, show similar patterns (Fig. 6A) and a higher number of differentially expressed genes (Table 2). We compared the RNA-seq data of these two mutants, which revealed that several potential pathogenicity genes are differentially regulated (Supplementary Fig. S9). The downregulated datasets of these mutants share 204 of 420 genes, 3 of which—MGG_01662 (4-aminobutyrate aminotransferase), MGG_02329 (isotrichodermin C-15 hydroxylase), and MGG_02378 (glutamate decarboxylase)—are known to be involved in pathogenicity of *M. oryzae* and other organisms (Guo et al. 2011; Kawahara et al. 2012). Further studies will be necessary to understand the role of MoRdRP1 in regulation of pathogenicity of the fungus.

We focused further attention on upregulated genes in the mutants *Δmodc12*, the double Dicers, *Δmordr2*, and *Δmoago3* because, collectively, these mutants showed the most varied sRNA profiles compared with the wild type (Figs. 2A, 3A, and 4A). We examined the upregulated genes in these mutants for commonalities. Minimal overlap was identified, with most of it being 19 genes shared between *Δmoago3* and the Dicer mutants (out of 114 and 102 genes, respectively) (Fig. 6B). Interestingly, 10 of these 19 genes have either a 3' or 5' end missing, indicating their likelihood as pseudogenes. Only 2 of the 19 have predicated functions as an endoglucanase (MGG_06069) and a methyltransferase (MGG_04036). Mutant *Δmodc12* and the double-Dicer mutants shared many upregulated genes, including six with likely or known roles in pathogenicity such as MGG_14068, coding for a putative FAD oxidoreductase, and MGG_10932 (coding ZNF1), known to be downregulated by chemical and physical signals (Oh et al. 2012). ZNF1 encodes a C2H2 zinc-finger transcription factor, which is essential for appressorium development in *M. oryzae* (Yue et al. 2016). Another important gene upregulated in the double-Dicer mutants is MGG_10315, which encodes the hydrophobin-like protein MPG1. *MPG1* is a well-characterized gene involved in pathogenicity of *M. oryzae* (Kershaw et al. 1998; Talbot et al. 1993). MGG_02065 (coding

Fig. 4. Small RNA (sRNA) profiling of the *Magnaporthe oryzae* wild type and Argonaute mutants. **A**, Proportion of total genome-matched sRNA (excluding transfer RNA [tRNA]-, ribosomal RNA [rRNA]-, small nuclear RNA [snRNA]-, and small nucleolar RNA [snoRNA]-matched reads) in each size class for the wild type (Wt) and mutant libraries. **B**, Distribution of sRNA reads of the wild type and mutants among different genomic elements normalized to tRNA-associated sRNA reads; nt = nucleotides. **C**, 5' Nucleotide preference for sRNA in the *M. oryzae* wild type and mutant libraries. **D**, 5' Nucleotide preference for sRNA matching repeats in the *M. oryzae* wild type and mutant libraries.

Fig. 5. Phenotypic analysis of the *Magnaporthe oryzae* wild type and RNA interference (RNAi) mutants. **A**, Colony morphology of the wild type and mutants grown on oatmeal media for 10 days. **B**, Colony diameters of the wild type and mutants (grown as per A) were measured and subjected to statistical analysis. Values are mean $\pm$ standard error of the mean (SEM) of three biological replicates. **C**, Production of conidia from the wild type (Wt) and mutants grown on oatmeal agar for 10 days. Values are mean $\pm$ SEM of three biological replicates. **D**, Pathogenicity test on barley leaves. Hyphal plugs from the wild type and mutants grown on complete media for 10 days were placed on 7-day-old barley leaves, and incubated in moistened chamber for 4 to 7 days. **B** and **C**, Bars with different letters are significantly different according to Tukey's honestly significant difference test ($P < 0.05$).

for kinesin light chain) (Villalba et al. 2008) and MGG_10027 (coding for calcium-transporting ATPase 1 [MoACU1]) (Benito et al. 2011) were upregulated in $\Delta modc12$ and $\Delta modc11/2$ mutants. MGG_04729 was upregulated in $\Delta modc12$ and double-Dicer mutants. Although this gene has no known function, it has reduced transcript abundance at 4 h postgermination in the *M. oryzae* *mst12* mutant (Park et al. 2002). Mst12 is a transcription factor similar to *steA* and coded by MGG_12958. Although we could not identify a negative relationship between sRNA and mRNA levels in these six genes for all mutants, we did note that, in the two mutants with reduced virulence ($\Delta rdp1$ and $\Delta ago3$), only two of the six pathogenicity genes (MGG_02065 and MGG_04729) were upregulated. The other four genes were downregulated, including the ZNF1 transcription factor and *MPG1*. These patterns could result directly from the loss of sRNA or as a secondary consequence.

Genes associated with differentially regulated sRNA clusters are proximal to repeat regions.

We wished to further examine whether we could identify any characteristics specific to genes associated with sRNA DRC. The $\Delta modc11$ deletion mutant has only two DRC, both of which are downregulated and both of which associate with a single, hypothetical gene, MGG_14670 (Table 1). Examination of 88 genes associated with downregulated sRNA DRC in $\Delta modc12$, the double-Dicer mutants, $\Delta mordrp2$, and $\Delta moago3$ showed that 41 of these genes are present within, are part of, or are in very close proximity to the repeat region (within 100 bp). A closer look at the remaining 47 genes showed that most of the sRNA derive from sense strands, consistent with products of mRNA degradation (Fig. 7A to C). Most of these genes also show lower transcript abundance, indicating that these sRNA are not involved in the regulation of this subset of genes, at least under our tested conditions.

RdRP-dependent sRNA require AGO for transcriptional regulation of IGR, and transcriptional regulation of repeats and IGR by sRNA.

To begin investigating the regulatory potential of sRNA, we compared the transcriptional impact of the loss of MoDcl2 or both Dicers, focusing on repeats and IGR, for loci with greater transcript abundance. Not surprisingly, the results were consistent with transcriptional regulation by sRNA at repeats (including LTR and GYPSY, and LINE and DNA-transposons) and IGR (Fig. 7D-E). While examining the most downregulated IGR-associated DRC of $\Delta mordrp2$ (Supplementary Table S4), we found that these regions generate abundant sRNA in strain 70-15, similar to repeat regions, which prompted us to further

examine these genomic regions. These IGR carry clusters of sRNA on both strands similar to repetitive elements, and 314 of them have homology to telomeric regions of *M. oryzae*, identified by BLAST searches. Interestingly, RNA-seq data identified transcripts from these regions that showed increased expression in the $\Delta moago3$ mutant (Fig. 8) compared with the wild type. The set of IGR-associated, downregulated DRC of $\Delta mordrp2$ shared only three clusters in common with $\Delta modc12$ (including double-Dicer mutants). Taken together, we can hypothesize that MoRdRP2 is involved in secondary amplification of sRNA from IGR, and MoAgo3 is involved in transcriptional regulation of IGR via sRNA; however this does not preclude the regulation of these IGR by an alternate mechanism. In 2015, Jeon and associates (2015) published the *M. oryzae* methylome, and we compared DRC from Figures 7 and 8 to their methylation data. Although we were unable to identify overlap between our dataset and their methylation data (data not shown), our comparison was only on a subset of data, thus warranting further examination of alternate regulatory mechanisms.

DISCUSSION

Our study provides new insights into the role in the plant-pathogenic ascomycete fungus, *M. oryzae*, of RNAi genes in the biogenesis and genomic distribution of endogenous sRNA, as well as in growth and pathogenicity. Our goal was to comprehensively analyze transcriptional profiles of mutants in RNAi biogenesis genes in this fungus, in order to better understand (i) transcriptional and posttranscriptional gene regulation in this fungus and (ii) which genes played the most critical roles in RNAi. We sequenced and analyzed sRNA and the mRNA transcriptome, and we determined growth and pathogenicity phenotypes of the wild type and 10 RNAi deletion mutants. Our findings demonstrate that MoDcl2, MoRdRP2, and MoAgo3 are primary contributors to sRNA biosynthesis and to the generation of different size classes of sRNA. These endogenous sRNA appear to be involved in the regulation of repeat regions and IGR. Though AGO are mainly involved in stability and functioning of sRNA, their involvement in biogenesis of miRNA of *Neurospora* spp. (Lee et al. 2010) and class III Ex-siRNA of *Mucor circinelloides* (Cervantes et al. 2013) have been reported.

sRNA regulate transcription in the intergenic and repeat regions of *Magnaporthe oryzae*.

Repetitive elements constitute approximately 10% of the genome of *Magnaporthe oryzae* (Okagaki et al. 2015). Activities of the transposons are believed to be controlled by RNAi mechanisms distinct from transcriptional gene silencing and siRNA-mediated gene silencing (Murata et al. 2007). In our study, sRNA associated with repeats are significantly downregulated in $\Delta modc12$, $\Delta mordrp2$, and $\Delta moago3$ deletion mutants, with a higher number of downregulated LTR- and GYPSY-associated DRC in the $\Delta mordrp2$ mutant (Table 1). This suggests the involvement of RdRP in the formation of dsRNA, a substrate for Dicer activity to produce sRNA as well as for secondary amplification of sRNA signals. In many organisms, including fungi, RdRP are involved in generation of dsRNA from single-stranded RNA, as well as production of secondary sRNA (Calo et al. 2012; Dang et al. 2011).

IGR constitute 38% of the genome (*Magnaporthe comparative* Sequencing Project, Broad Institute of Harvard and MIT), and sRNA associated with IGR constituted 47% of the total reads (Fig. 2B). Because the majority of the sRNA were generated from IGR, we checked for the possibility of miRNA but were unable to identify miRNA in *M. oryzae*, consistent with Gowda and associates (2010) and Nunes and associates (2011).

Table 2. Summary of differentially expressed genes among various RNA interference mutants

Mutants ^a	Number of genes		
	Total differentially expressed	Upregulated	Downregulated
$\Delta modc11$	21	13	8
$\Delta modc12$	43	14	29
$\Delta modc11/2$	105	94	11
$\Delta modc12/1$	35	12	23
$\Delta mordrp1$	420	91	329
$\Delta mordrp2$	17	10	7
$\Delta mordrp3$	9	8	1
$\Delta moago1$	69	23	46
$\Delta moago2$	45	21	24
$\Delta moago3$	409	114	295
Total	706	279	457

^a Mutants compared with the wild type.

Moreover, transcription was also occurring in these regions, at least to some extent (Fig. 7). This raises the possibility that transcription of IGR is effectively controlled by sRNA. The majority of IGR in which sRNA are reduced in a MoDcl2 mutant have repeat-like features (i.e., sRNA on both strands) and homology to telomeric regions. Telomeres are essential for chromosome function and genome integrity by preventing chromosome shortening (Peuscher and Jacobs 2011) and protecting the DNA from exonucleolytic activity (Zakian 1996). The subtelomeric gene families of microbial eukaryotes are often involved in niche adaptation by switching the expression of surface proteins (Sunkin and Stringer 1996). Telomeres of *M. oryzae* are highly variable regions, and novel telomeres arise frequently during culturing (Farman 2007; Rehmeier et al. 2006). Only a few of the terminal genes are duplicated in strain 70-15, used in this study, making it difficult for the fungus to use switching mechanisms to evade the host plant's surveillance mechanism (Farman 2007). Moreover, *M. oryzae* termini are also proposed to have subtle roles in host adaptation (Rehmeier et al.

2006). Our study reveals a prevalence of downregulated DRC associated with repeats as well as IGR that show homology to telomeric regions. This observation suggests a role for the endogenous sRNA pathway in protecting the *M. oryzae* genome by regulating the IGR, telomeric, and repeat regions. In fission yeast, RNAi machinery is required for proper regulation of chromosome dynamics during mitosis and meiosis, with RNAi mutants showing aberrant telomere clustering during meiotic prophase (Hall et al. 2003).

Preference for 5'-U sRNA.

RNAi by homology-dependent transcriptional cleavage or posttranscriptional silencing is carried out by binding of sRNA to AGO in animals, an association primarily based on the structural features of precursor duplexes (Steiner et al. 2007; Tomari et al. 2007), and in plants by the nucleotide occupying the 5' position of sRNA (Mi et al. 2008). In fungal species, a 5' nucleotide preference for U has been reported in *M. oryzae* (Nunes et al. 2011; Raman et al. 2013), *N. crassa* (Lee et al.

Fig. 6. Transcriptome profiling of RNA interference (RNAi) deletion mutants by RNA sequencing. **A**, Heat map of $\log_{10}$ fragments per kilobase per million (FPKM) + 1 expression levels of differentially expressed (706) genes in the wild type and RNAi deletion mutants. **B**, Venn diagram showing overlap between upregulated genes of $\Delta modcl2$ (including double Dicer), $\Delta mordrp2$, and $\Delta moago3$ mutants.

2010), *S. pombe* (Bühler et al. 2008), *S. castelleii*, *Kluveromyces polysporus* (Drimmenberg et al. 2009), and *Mucor circinelloides* (Nicolas et al. 2010). In *N. crassa*, efficient loading onto AGO is dictated by a preference for 5'-U sRNA, which are the product of Dicer2 processing; whereas, in *S. pombe*, the loading

preference for 5'-U sRNA in the AGO is attributed to higher stability of the sRNA (Bühler et al. 2008). Our study showed a reduction in 5'-U sRNA associated with protein coding regions in the $\Delta modc12$, double Dicers, and $\Delta mordrp2$ mutants, suggesting that they are being produced by MoDc12. Additionally, we

Fig. 7. Relationship between gene-specific, intergenic (IG), and repeat-associated small RNA (sRNA) and messenger RNA (mRNA). Plotting of log₂ fold changes of sRNA and RNA sequencing (RNA-seq) data for genes associated with downregulated differentially regulated cluster (DRC) from static clustering of **A**, $\Delta modc12$ (including double Dicer mutants) versus wild-type; **B**, $\Delta mordrp2$ versus wild-type; and **C**, $\Delta moago3$ versus wild-type. sRNA and RNA-seq data for downregulated DRC associated with **D**, LTR (long terminal repeat) and Gypsy (LTR type retrotransposon) and **E**, IG for $\Delta modc12$ (including double Dicer mutants) versus wild-type.

observed an absence of reduction in the $\Delta moa3$ mutant, indicating that the 5'-U sRNA are still being produced, just not loaded into MoAgo3. On the other hand, there was a slight reduction in 5'-U sRNA in the $\Delta moa3$ mutant associated with IGR and repeat regions, and the size class of sRNA found in this mutant was different compared with the wild type and the other mutants. Together, these data indicate that MoAgo3 might contribute to the biogenesis of sRNA but is not essential. In *Arabidopsis*, mutation of the *AGO4* gene resulted in reduction but not elimination of longer (>25 nt) siRNA, demonstrating that it is important but not essential for smaller siRNA (Blevins et al. 2015). This further warrants the study of AGO-associated sRNA by immunoprecipitation of AGO-bound sRNA to understand which sRNA are recruited into the AGO-containing RISC complex (Wang et al. 2011; Wang et al. 2016).

Regulation by gene-specific sRNA.

In fungi, there are demonstrated examples of transcriptional gene regulation by gene-specific sRNA (siRNA, miRNA, or miRNA), as exemplified by exonic-siRNA of *M. circinelloides* (Nicolas et al. 2010) and miRNA of *N. crassa* and *Penicillium marneffei* (Lau et al. 2013; Lee et al. 2010). In *F. graminearum*, Fg-miRNA-4 negatively regulates the expression of its target gene (Chen et al. 2015). The role of MoDcl1 in *Magnaporthe oryzae* is believed to be insignificant (Nakayashiki et al. 2006). Our recent work revealed that MoDcl1 might have a small role in gene regulation, evident through increased expression of approximately 10 genes in the $\Delta modcl1$ mutant (Raman et al. 2013). Here, we found that upregulated genes in the $\Delta modcl1$ mutant also had increased sRNA accumulation, suggesting that degradation of these transcripts leads to increased accumulation of sRNA in this mutant. The results deciphered from sRNA sequencing of the wild type and *modcl* mutants indicated that MoDcl2 is primarily involved in sRNA biogenesis. Based on results from this study, we speculate that increased expression observed in RNAi deletion mutants is likely due to the secondary consequences of RNAi loss, not because of direct targeting by sRNA (Fig. 7).

Regulation of pathogenicity by RNAi genes.

We analyzed all RNAi biogenesis mutants by pathogenicity assays and observed reduced virulence in $\Delta moa3$ and $\Delta mordrp1$. Interestingly, even during wound assays, $\Delta moa3$ and $\Delta mordrp1$ were unable to infect barley, and both mutants demonstrated reduced conidial production. These important infection and pathogenicity phenotypes are reflected in the RNA-seq dataset, where a subset of downregulated genes is known to be involved in virulence. A notable gene in the datasets of $\Delta moa3$ and $\Delta mordrp1$ is MGG_01662, coding for 4-aminobutyrate aminotransferase. Previous research showed that deletion of this gene did not alter conidial germination and appressorium formation but did reduce pathogenicity (Guo et al. 2011). Furthermore, this gene is reported to be positively regulated by MoAPI1, a transcription factor that regulates reactive oxygen species scavenging in *M. oryzae* (Guo et al. 2011). Another downregulated gene from the datasets of $\Delta moa3$ and $\Delta mordrp1$ was MGG_02329, coding for isotrichodermin C-15 hydroxylase. A prior study found that this gene was upregulated in *M. oryzae*-infected rice leaves (Kawahara et al. 2012). A similar gene from *F. graminearum* was upregulated in crown rot and head blight (Stephens et al. 2008)-infected wheat. MGG_02378 coding glutamate decarboxylase (GLD2) is Tra1p-dependent gene, and Tra1p is required for normal adhesion, germination, appressorium formation, and virulence (Breth et al. 2013). Surprisingly, the tRNA-associated sRNA abundances of both $\Delta mordrp1$ and $\Delta moa3$ mutants were reduced compared with the wild type (data not shown), and it is noteworthy that the abundance of tRNA-associated sRNA increased in appressoria compared with

mycelia of the wild type (Nunes et al. 2011). Based on our data, the $\Delta mordrp1$ mutant likely does not have a prominent role in endogenous sRNA pathways; however, the $\Delta mordrp1$ mutant may be an interesting candidate for future studies to establish the role of MoRdRP1 in regulating the pathogenicity of the fungus through regulation of tRNA-associated sRNA. Together, our findings indicate that the regulation of virulence genes by RNAi is controlled, at least in part, by the downregulation of these transcripts. Other control mechanisms could include passage of sRNA from fungus to host, as in the case of the gray mold *B. cinerea*; however, it remains to be determined whether this is the case for *M. oryzae*. Contrary to our findings, in *Mucor circinelloides*, endogenous sRNA are reported to be involved in transcriptional regulation of mRNA (Nicolas et al. 2010). Further, miRNA and miRNA are involved in gene regulation in fungal species (Chen et al. 2015; Lee et al. 2010).

Concluding remarks.

The present study indicates that the *Magnaporthe oryzae* sRNA transcriptome as well as its regulatory role are different from other fungal species. Moreover, MoDcl2, MoRdRP2, and MoAgo3 are the primary factors involved in endogenous siRNA biogenesis in *M. oryzae*. Examination of deletion mutants of RNAi genes indicates that subsets of IGR and repeats are transcriptionally regulated by sRNA. Future studies on AGO-bound sRNA as well as sRNA produced by RNAi mutants in planta will provide a better understanding of functions and roles of sRNA in shaping fungal pathogenicity. Given that, in certain plant-pathogen interactions, sRNA act as effectors to suppress host immunity (Weiberg et al. 2013), deeper insights into the sRNA suite of plant-pathogenic fungi will reveal the role of sRNA in the evolutionary arms race between host and plant pathogen.

MATERIALS AND METHODS

Fungal strains and growth conditions.

M. oryzae wild-type strain 70-15 and targeted deletion mutants of RNAi genes in 70-15 background were used in this study. Filter paper stocks stored at -20°C were inoculated onto OM plates (oatmeal at 50 g/liter and agar at 15 g/liter) for germination and to establish starter cultures. Fungus grown on OM plates were transferred to complete medium (10 g of sucrose, 6 g of yeast extract, 6 g of casamino acid, and 1 ml of trace elements per liter) plates and, subsequently, to complete liquid medium. Five-day-old liquid grown fungus was harvested under sterile conditions and washed with sterile distilled water, frozen immediately in liquid nitrogen, and stored at -80°C for further analyses.

Fig. 8. Common intergenic region (IG)-associated loci between down-regulated small RNA (sRNA) clusters of $\Delta mordrp2$ and upregulated RNA sequencing (RNA-seq) clusters of $\Delta moa3$.

Targeted disruption of genes involved in sRNA biogenesis and mutant screening.

Generation of targeted disruption mutants of *MoDcl1*, *MoDcl2*, single and both Dicers, and *MoRdRP2* genes are described by Raman and associates (2013). Targeted disruption mutants of *MoRdRP1*, *MoRdRP3*, and *AGO* were generated in similar manner. Gene deletions were confirmed by Southern blots and reverse-transcription polymerase chain reaction. Primers used in this study are listed in Supplementary Table S5. The mutant corresponding to the *MoAgo3* gene (MGG_01294) was processed for sRNA sequencing in an experiment separate from the other mutants, due to the fact that the original mutant was unconfirmed and, thus, we later remade it. The other mutants, along with the wild type, were processed together for sRNA sequencing in experiment I, while the $\Delta moago3$ mutant was processed with the wild type at a later date in experiment II. The wild type is the same material for both experiments.

RNA isolation, sRNA and mRNA library construction, and data analysis.

Total RNA was isolated using Trizol reagent (Invitrogen, New York) according to the manufacturer's instructions. The quality of the RNA was checked by agarose gel electrophoresis and NanoDrop ND-1000. The concentration of RNA was checked by Qubit (Invitrogen). sRNA libraries were prepared from total RNA samples using a TruSeq Small RNA Preparation kit following Illumina protocols (Illumina, San Diego, CA, U.S.A.) using 1 μ g of total RNA, and size selected to eliminate intact tRNA and rRNA. Sequences obtained from the sequencer were processed as described earlier (Raman et al. 2013). In brief, adapters were removed from the resulting sequences and matched the *M. oryzae* genome (version 8 assembly) from the Broad Institute. tRNA-, rRNA-, snRNA-, or snoRNA-matched reads were removed from the total reads. From the total genome-matched reads, distinct genome-matched reads were calculated based on the hits. The abundance of each read was normalized to transcripts per 5 million. To compare the different libraries, the entire genome was segmented into 500-bp windows or clusters based on the proximity-based algorithm (Lu et al. 2005) (described in the Results section). Clusters showing ≥ 10 -fold differences between two or more libraries were selected for further cluster analysis. Repeat analysis was performed using programs RepeatMasker (Smit et al. 1996-2000), etandem, and einverted (Rice et al. 2000).

mRNA libraries were prepared from total RNA samples using the TruSeq RNA sample preparation kit following manufacturer's instructions (Illumina). Briefly, each total RNA sample (4 μ g) was treated with DNase and enriched for mRNA using oligo(dT) tags. From these enriched samples, cDNA libraries were constructed by following steps such as fragmentation, end repair, A tailing, adapter ligation, and library amplification. Individually barcoded libraries were pooled and sequenced on an Illumina HiSeq2000 at the Delaware Biotechnology Institute. The short reads were processed with Tophat (v2.0.1) (Kim et al. 2013) using default options and mapped to the reference genome downloaded from the Broad website using Bowtie2 (Langmead and Salzberg 2012). Cufflinks program (version 2.2.0) was used for counting and expression scoring (Trapnell et al. 2010). Cuffdiff2 was used to determine expression levels (fragments per kilobase per million mapped reads) and differential expression between the wild type and various RNAi mutants using default options (Trapnell et al. 2013). The results of Cuffdiff2 analyses were visualized by the R program CummeRbund v2.1 (Goff et al. 2013).

Genomic coordinates for repeats and IGR extracted from an internally generated database were used to make 'bed' files, repeats, and IGR. Differential RNA expression analysis for these regions was carried out using RepEnrich (Criscione et al.

2014) and EdgeR (Robinson et al. 2010) by following the steps outlined by Criscione and associates (2014) and De Cecco and associates (2013). All data generated in this study can be found at the National Center for Biotechnology Information GEO GSE81640.

Detached-leaf assay.

Conidia from *M. oryzae* 70-15 and mutants grown on OM plates for 10 days were harvested using water. Concentration of the conidial suspension was adjusted to 1×10^5 spores/ml. The suspension (25 μ l) was placed on barley leaves harvested from 7-day-old plants, placed on a moistened filter paper, and placed in a closed chamber kept under darkness overnight. The next day, spore suspensions were wiped off and leaves were exposed to continuous light for 4 to 7 days. For *RdRP* and *AGO* mutants, inoculation of leaves with hyphal plugs was carried out.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Broad Institute *Magnaporthe* comparative genomics database:
https://www.broadinstitute.org/annotation/genome/magnaporthe_comparative/MultiHome.html
- Broad Institute website: <https://www.broadinstitute.org/>
- JGI MycoCosm database, *Cryphonectria parasitica* EP155 v2.0:
<http://genome.jgi.doe.gov/Crypa2/Crypa2.home.html>
- Joint Genome Institute (JGI) MycoCosm database, *Mucor circinelloides* CBS277.49 v2.0: <http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html>
- Magnaporthe* Next-Gen Sequence sRNA (new) database:
https://mpss.danforthcenter.org/dbs/index.php?SITE=mg_sRNA_new
- Magnaporthe* Next-Gen Sequence sRNA database:
https://mpss.danforthcenter.org/dbs/index.php?SITE=mg_RNAseq
- MicroRNA Discovery By Deep Sequencing MIREAP tool:
<https://sourceforge.net/projects/mireap/>